for 1 minute. The gentamicin fractions appear as reddish zones. The zone furthest from the origin is gentamicin C_1, the one closest is gentamicin C_{1a}, and the middle zone is gentamicin C_2. Cut the corresponding zones out of the other unsprayed half of the sheet. Cut each portion of the sheet thus obtianed into small strips and put those from each zone into a separate 125-milliliter glass-stoppered flask. Add 50 milliliters of 0.1*M* potassium phosphate buffer, pH 8, to each flask and swirl the flask mechanically for 30 minutes. Decant the solution from each flask into separate test tubes and allow the paper to settle. Pipet 4 milliliters of each clear solution into a 25-milliliter volumetric flask and make to volume with the pH 8 buffer. Assay these solutions as directed in paragraph (b)(1) of this section.

(vi) *Calculations.*

$$\text{Total gentamicins} = \frac{\text{Assay of } C_1 \text{ fraction}}{0.786} + \frac{\text{Assay of } C_2 \text{ fraction}}{1.023} + \frac{\text{Assay of } C_{1a} \text{ fraction}}{0.977}$$

$$\text{Percent of gentamicin } C_1 = \frac{\text{Assay of } C_1 \text{ fraction}}{0.786} \times \frac{100}{\text{Total gentamicins}}$$

$$\text{Percent of gentamicin } C_2 = \frac{\text{Assay of } C_2 \text{ fraction}}{1.023} \times \frac{100}{\text{Total gentamicins}}$$

$$\text{Percent of gentamicin } C_{1a} = \frac{\text{Assay of } C_{1a} \text{ fraction}}{0.977} \times \frac{100}{\text{Total gentamicins}}$$

Where:

The assays are expressed in terms of the microgram equivalents of gentamicin; and

The factors 0.786, 1.023, and 0.977 represent the activities of gentamicins C_1, C_2, and C_{1a} relative to the gentamicin activity of the gentamicin master standard.

(9) *Identity.* Proceed as directed in § 436.211 of this chapter, using a 0.5 percent mixture of the sample in a potassium bromide disc prepared as described in paragraph (b)(2) of that section.

[39 FR 19046, May 30, 1974, as amended at 50 FR 19919, May 13, 1985]

§ 444.30 Kanamycin sulfate.

(a) *Requirements for certification*—(1) *Standards of identity, strength, quality, and purity.* Kanamycin sulfate is the sulfate salt of a kind of kanamycin or a mixture of two or more such salts. It is so purified and dried that:

(i) Its potency on an anhydrous basis is not less than 750 micrograms of kanamycin per milligram.

(ii) [Reserved]

(iii) Its loss on drying is not more than 4 percent.

(iv) Its pH is an aqueous solution containing 10 milligrams per milliliter is not less than 6.5 and not more than 8.5

(v) Its residue on ignition is not more than 1.0 percent.

(vi) It gives a positive identity test for kanamycin.

(vii) It contains not more than 5.0 percent kanamycin B.

(viii) It is crystalline.

(2) *Labeling.* It shall be labeled in accordance with the requirements of § 432.5(b) of this chapter.

(3) *Requests for certification; samples.* In addition to complying with the requirements of § 431.1 of this chapter, each such request shall contain:

(i) Results of tests and assays on the batch for potency, loss on drying, pH, residue on ignition, identity, kanamycin B content, and crystallinity.

(ii) Samples required on the batch: 10 packages, each containing approximately 500 milligrams.

(b) *Tests and methods of assay*—(1) *Potency.* Proceed as directed in § 436.106 of this chapter, preparing the sample for assay as follows: Dissolve an accurately weighed sample in sufficient sterile distilled water to give a stock solution of convenient concentration. Further dilute an aliquot of the stock solution with sterile distilled water to the reference concentration of 10 micrograms of kanamycin per milliliter (estimated).

(2) [Reserved]

(3) *Loss on drying.* Proceed as directed in § 436.200(b) of this chapter.

(4) *pH.* Proceed as directed in § 436.202 of this chapter, using a solution containing 10 milligrams per milliliter.

(5) *Residue on ignition.* Proceed as directed in § 436.207(a) of this chapter.

(6) *Identity.* Dissolve about 10 milligrams of kanamycin sulfate in 1 milliliter of water, and add 1 milliliter of a 1:500 solution of triketohydrindene hydrate in normal butyl alcohol; then add 0.5 milliliter of pyridine. Heat in a steam bath for 5 minutes and add 10 milliliters of water; a deep-purple color is produced.

(7) *Kanamycin B content*—(i) *Cylinders* (*cups*). Use cylinders described under § 440.80a(b)(1)(i) of this chapter.

(ii) *Culture medium.* Use ingredients that conform to the standards prescribed by the U.S.P. or N.F. Make agar for the base and seed layers as follows:

Peptone	6.0 gm.
Yeast extract	3.0 gm.
Beef extract	1.5 gm.
Agar	15.0 gm.
pH 7.8 to 8.0 after sterilization.	
Distilled water, q.s	1,000.00 ml.

(iii) *Working standard.* Dissolve a suitable quantity of the kanamycin sulfate working standard, accurately weighed, in 0.1*M* potassium phosphate buffer, pH 8.0, to give a concentration equivalent to 1.0 milligram of kanamycin per milliliter.

(iv) *Preparation of sample.* To 100 milligrams, accurately weighed, of kanamycin sulfate in a suitable container (such as a 7.5-milliliter serum vial) add 5.0 milliliters of 6*N* hydrochloric acid, and tightly close the container. Heat in a water bath at 100° C. for 1 hour and cool. Add 4 milliliters of 6*N* sodium hydroxide, then dilute with sterile 0.1*M* potassium phosphate buffer, pH 8.0, to obtain a concentration of the equivalent of 1 microgram of kanamycin per milliliter (estimated).

(v) *Preparation of test organism.* Use *Bacillus subtilis* (ATCC 6633)[1] prepared as described in § 436.103 of this chapter, using method 2.

(vi) *Preparation of plates.* Add 21 milliliters of the agar prepared as described in paragraph (b)(7) of this section to each Petri dish (20 millimeters × 100 millimeters). Distribute the agar evenly in the plates and allow to harden. Use the plates the same day they are prepared. Add 4.0 milliliters of the fresh daily inoculum described in paragraph (b)(7)(iv) of this section to each plate, tilting the plates back and forth to spread the inoculated agar evenly over the surface.

(vii) *Standard curve.* Prepare on the day of testing in 0.1*M* potassium phosphate buffer, pH 7.8 to 8.0, from the standard stock solution, sufficient volumes of the following concentrations: 0.64, 0.8, 1.0, 1.25, and 1.56 micrograms per milliliter. The 1.0 microgram-per-milliliter solution is the reference point of the standard curve. On each of three plates fill three cylinders with the 1.0 microgram-per-milliliter standard and the other three cylinders with the concentration under test. Thus, there will be thirty-six 1.0-microgram determinations for each of the other points on the curve. After the plates have incubated read the diameters of the circles of inhibition. Average the readings of the 1.0 microgram-per-milliliter concentration and the readings of the concentration test for each set of three plates and average also all 36 readings of the 1.0 microgram-per-milliliter concentration. The average of the 36 readings of the 1.0 microgram-per-milliliter concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 1.0 microgram-per-milliliter reading for that set of three plates were the same as the correction point. Thus, if in correcting the 0.8-microgram concentration, the average of the 36 readings of

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

the 1.0 microgram-per-milliliter concentration is 16.5 millimeters and the average of the 1.0 microgram-per-milliliter concentration of this set of three plates is 16.3 millimeters, the correction is +0.2 millimeter. If the average readings of the 0.8 microgram-per-milliliter concentration of these same three plates is 15.9 millimeters, the corrected value is 16.1 millimeters. Plot these corrected values, including the average of the 1.0 microgram-per-milliliter concentration, on 2-cycle semilogarithmic paper, using the concentration in micrograms per milliliter as the ordinate and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points, either by inspection or by means of the following equations:

$$L = \frac{3a + 2b + c - e}{5}$$

$$H = \frac{3e + 2d + c - a}{5}$$

where:

L=Calculated zone diameter for the lowest concentration of the standard curve;

H=Calculated zone diameter for the highest concentration of the standard curve;

c=Average zone diameter of 36 readings of the 1.0 microgram-per-milliliter standard;

a, b, d, e=Corrected average values for the 0.64, 0.8, 1.0, 1.25, and 1.56 micrograms-per-milliliter solutions, respectively.

Plot the values obtained for *L* and *H* and connect with a straight line.

(viii) *Assay.* Place six cylinders on the inoculated agar surface in each Petri dish prepared as described in paragraph (b)(7)(vi) of this section, so that they are at approximately 60° intervals on a 2.8-centimeter radius. Use three plates for each sample. Fill three cylinders on each plate with the 1.0 microgram-per-milliliter standard and three cylinders with the 1.0 microgram (estimated)-per-milliliter sample, alternating standard and sample. Incubate plates for 16 hours to 18 hours at 32° C. to 35° C., and measure the diameter of each circle of inhibition.

(ix) *Estimation of kanamycin B content.* Average the zone readings of the standard and average the zone readings of the sample on the three plates used. If the sample gives larger average zone size than the average of the standard, add the difference between them to the 1.0-microgram zone size of the standard curve. If the average value is lower than the standard value, subtract the difference between them from the 1.0-microgram value on the curve. From the curve, read the kanamycin potencies corresponding to these corrected values of zone sizes. Multiply the observed potency by 100 and divide by 126 to obtain a value representing the potency in terms of the milligram equivalent of kanamycin B. The calculated amount of kanamycin B is not more than 5 percent of the content of kanamycin found in paragraph (b)(1) of this section.

(8) *Crystallinity.* Proceed as directed in § 436.203(a) of this chapter.

[39 FR 19046, May 30, 1974, as amended at 50 FR 19919, May 13, 1985]

§ 444.30a Sterile kanamycin sulfate.

(a) *Requirements for certification*—(1) *Standards of identity, strength, quality, and purity.* Kanamycin sulfate is the sulfate salt of a kind of kanamycin or a mixture of two or more such salts. It is so purified and dried that:

(i) Its potency on an anhydrous basis is not less than 750 micrograms of kanamycin per milligram.

(ii) It is sterile.

(iii) [Reserved]

(iv) It is nonpyrogenic.

(v) Its loss on drying is not more than 4 percent.

(vi) Its pH in an aqueous solution containing 10 milligrams per milliliter is not less than 6.5 and not more than 8.5.

(vii) Its residue on ignition is not more than 1.0 percent.

(viii) It gives a positive identity test for kanamycin.

(ix) It contains not more than 5.0 percent kanamycin B.

(x) It is crystalline.

(2) *Labeling.* It shall be labeled in accordance with the requirements of § 432.5(b) of this chapter.

(3) *Requests for certification; samples.* In addition to the requirements of § 431.1 of this chapter, each such request shall contain: